A glucose agar, N-free, and a similar liquid medium were inoculated with a sample of the sand. After a week some white colonies appeared on the surface of the agar. At the end of three weeks, the colonies became yellow. The inoculated liquid medium became turbid after the same incubating period; there was no pellicle formation. The medium also became slightly acid; Bromothymol Blue gave a yellow colour but the acidity was not enough to give a red colour with Methyl Red, thus indicating a pH between 5 and 6. The microscopic examinations showed the presence of nearly pure cultures of large coccoid cells containing prominent refractive bodies. When stained with Methylene Blue the cytoplasm of these cells showed blue with large colourless globules.

As the mineral solution used in the pot experiments had an acid pH slightly above 5·5, it was natural to consider the possibility of the acid-tolerant *Beijerinckia* being present. However, a close examination of cells showed that the morphology of this organism differed from that of *Beijerinckia*.* They were not rods, but coccoid cells like those of *Azotobacter*, differing from *Azotobacter chroococcum*, *Azotobacter vinelandii*, and *Azotobacter agilis* in the presence of fatty bodies and in cultural characters, especially the absence of pellicle formation in liquid medium. Winogradsky (1938) has noted transitory lipoid bodies in the precystic stage of *Azotobacter vinelandii*, but in our organism the bodies are permanent. Lipman (1903) has already recorded lipoid bodies in *Azotobacter beijerinckii* from which our organism differs by its tolerance to acid conditions.

It is premature to discuss the taxonomic position of this organism and further cytological and physiological studies are necessary. The occurrence of the acid-tolerant organism was of considerable interest and a survey of soils in the Sydney District was undertaken to determine the natural occurrence of the organism.

METHODS.

Medium: The original medium used by Starkey and De (1939) to isolate their organism, was a mineral basic medium with sucrose as organic matter. Derx (1950) suggested using an acid medium at pH 5 to prevent the growth of *Azotobacter*. This medium, however, hydrolyses the agar during sterilization. The medium finally adopted is Winogradsky's standard medium with 1 part in 10,000 of humus and 1% of glucose at pH 7·2. Humus was extracted by NaOH and dialysed first against tap water for twenty-four hours and then against distilled water for twenty-four hours. When the solid medium is used 2% of washed agar is added.

Qualitative detection. 0·1 g. fresh soil is first sprinkled on the surface of the agar medium and 5 g. is inoculated in 45 c.c. of liquid medium. The incubation is at 30°C. After a few days, the colonies or liquid cultures are examined. Cultures are kept in the incubator for one month before being considered negative.

MACROSCOPIC CHARACTER OF CULTURE.

Agar medium: Colonies are large (2 mm. in diameter. After three or four weeks their diameter may be 10 mm.). They are first white, becoming slightly yellow or grey. Some strains develop a dark chocolate colour different from the almost black colour of *Azotobacter chroococcum*. Microscopic examination is necessary in doubtful cases.

Liquid medium: The cultures start with a uniform turbidity of the medium. A close examination shows that the suspension of cells is rough and agglutinated. In old cultures there is no pellicle formation on the surface of the liquid medium. Sometimes a ring is formed. The ring may be wide enough to cover the centre (in a test tube), but even in this case, after gentle shaking, the mass of bacterial cells falls slowly to the bottom of the medium which becomes cloudy. This development is quite different from the characteristic pellicle formation of *Azotobacter*. In a mixed culture with *Azotobacter* the liquid medium is much more difficult to use because the whole picture is dominated by *Azotobacter*. In a mixed culture microscopic examination shows the presence of our organism. When the liquid culture alone gives a positive test it is always considered as a doubtful case. The liquid culture is only considered positive when a transfer to a solid medium gives a characteristic culture.

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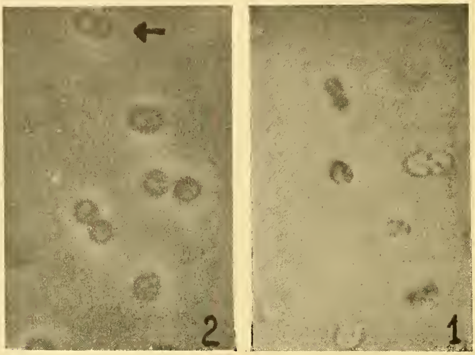
* *Beijerinckia* strains were kindly supplied by Professor Derx and Miss J. T. de Vries, to whom we are much indebted.

Microscopic Character of Cells.

The typical difference between our organism and other *Azotobacter* is the presence of large fatty bodies, not stainable by the aniline dyes or iodine. When a culture is examined in the presence of Methylene Blue (aqueous solution) under the high-power lens, large colourless globules can be seen very clearly in the cells against the blue cytoplasm. One drop of culture was suspended in one drop of 2 parts per 1,000 of Methylene Blue. When the culture showed a positive test, the slide was dried, washed with alcohol to remove the Methylene Blue and stained with Gram stain.* Our organism (Gram negative) shows red cytoplasm, including large colourless fatty bodies which differentiated it from other species of *Azotobacter*.



Text-figures 1, 2.

1.—Photomicrograph (× 1,900). Living cells in Methylene Blue aq. solution. White spots are lipoid bodies.

2.—Photomicrograph (× 1,900). Photograph taken with phase contrast microscope. Arrow indicates one *Beijerinckia*-like cell.

Quantitative Estimation: The technique used has been described previously (Tchan, 1952). The combination of a liquid and a solid medium for the estimation of our organism gives good results when it is the only non-symbiotic aerobic N-fixing bacterium present. In a mixed culture, when *Azotobacter* is present, it is not very difficult to distinguish the colonies of our organism from *Azotobacter* colonies on the agar medium, but in the liquid medium, as already mentioned, the estimation is not always as reliable as in agar medium. The liquid medium is therefore only used as a control of the agar medium.

pH Determination: Samples are first tested colorimetrically to determine their approximate pH. If the soil gives a positive growth of our organism an electrometric pH determination is carried out with a glass electrode.

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* (1) Crystal violet 10 g. Ammonium oxalate 4 g. 95% alcohol 100 c.c. Water 400 c.c.
(2) Iodine 1 g. KI 2 g. 95% alcohol 25 c.c. Water 100 c.c.
(3) Iodine solution as above 5 c.c. Alcohol 95% 95 c.c.
(4) Erythrosin 1 g. Phenol 5 g. Water 100 cc.

Stain 1 minute with crystal violet. Wash with tap water. Stain 1 minute with iodine (2) solution. Wash with iodine solution (3) until no more violet colour can be removed. (If the slide is still violet or blue, the destaining operation may be continued 20 or 30 minutes.) Wash with water. Counterstain with Erythrosin. Gram positive organisms are violet or blue and Gram negative organisms are red. Soil particles are colourless.

Humus Determination: The estimation of humus content is only made with samples giving a positive growth of our organism. The technique adopted is Demolon's method (1944). This permits comparative studies of the relationship between humus content and *Azotobacter* population of soils, and enables us to make a comparison with the results of Chalaust (1948) in France.

RESULTS.

The results of the survey are summarized in Tables I and II. Table I gives all soils tested, arranged in order of pH. Table II describes in more detail the soils giving a positive growth of our organism or of *Azotobacter*.

TABLE I.

Sample Positive or Negative.	Date.	Collected By.	pH.	Soil Type.	Locality.
–	16.5.51	Crommelin.	3·5	Sand.	Warrah Reserve, near Woy Woy.
–	16.5.51	Crommelin.	4·3	Sand (orchard).	Warrah Reserve, near Woy Woy.
–	16.5.51	Crommelin.	4·5	Sand.	Warrah Reserve, near Woy Woy.
–	23.4.51	McKee.	4·5	Sand, virgin soil.	Northbridge.
–	20.4.51	Beadle.	5·0		New England.
–	25.4.51	Tchan.	5·0	Sandstone in cultivated soil.	Mosman.
–	25.4.51	Tchan.	5·0	Sandstone in cultivated soil.	Mosman.
–	30.4.51	Beadle.	5·0	Laterite.	French's Forest.
–	2.5.51	McLuckie.	5·0	Sandstone.	Leura.
–	2.5.51	McLuckie.	5·0	Sandstone.	Leura.
–	10.7.51	Baas-Becking.	5·0	Sandstone.	National Park.
–	10.7.51	Baas-Becking.	5·0	Sandstone.	National Park.
–	20.4.51	Edenborough.	5·0	Podsol sand.	Lindfield.
–	20.4.51	Edenborough.	5·5	Podsol sand.	Lindfield.
–	20.4.51	Beadle.	5·5	Shale podsol.	Chatswood.
–	23.4.51	McKee.	5·5	Garden.	Northbridge.
–	25.4.51	Tchan.	5·5	Garden soil.	Mosman.
–	25.4.51	Tchan.	5·5	Garden soil.	Mosman.
–	25.4.51	Swaby.	5·5		Hurstville.
	16.5.51	Crommelin.	5·5		Warrah.
	16.5.51	Beadle.	5·5	Peat.	Guyra, New England.
	16.5.51	Beadle.	5·5	Beech forest.	Guyra.
+	2.5.51	Bruck.	5·5	Sand.	Rose Bay.
+	7.3.51	Botany School.	5·5	River sand.	Nepean River.
+	20.4.51	Edenborough.	5·62	Podsol sand.	Lindfield.
+	25.4.51	Chalmers.	5·6	Clayey garden.	Eastwood.
+	10.7.51	Rivera.	5·65	Rainforest podsol.	Mt. Keira.
+	20.4.51	Edenborough.	5·9	Podsol (sand).	Lindfield.
–	23.4.51	Mercer.	6·0	Sandstone.	Fitzroy Hills.
–	16.5.51	Beadle.	6·0	Alluvial soil.	New England.
–	16.5.51	Beadle.	6·0		New England.
–	30.7.51	Tchan.	6·0	Residual laterite.	Newport.
–	16.5.51	Beadle.	6·0	Chocolate soil.	New England.
+	30.4.51	Fraser.	6·2	Sand.	Wollstonecraft.
–	30.7.51	Tchan.	6·2	Garden.	Newport.
+	20.4.51	Hindmarsh.	6·4	Sand.	Rose Bay.
+	25.4.51	Robertson.	6·4	Shale.	Haberfield.
–	30.7.51	Tchan.	6·7	Podsol.	Newport.
–	6.6.51	Beadle.	7·0		
+	23.4.51	Thornton.	7·0	Sand.	Homebush.
+	23.4.51	Thornton.	7·0	Sand.	Homebush.
–	16.5.51	Beadle.	7·0		New England.
+	7.5.51	Everson.	7·0	Garden.	Goulburn.
–	20.4.51	Edenborough.	7·0	Podsol sand.	Lindfield.
–	26.4.51	Burges.	8·0	Clayey garden.	Ryde.